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Characterizations and anti-tumor activities of three acidic polysaccharides from *Angelica sinensis* (Oliv.) Diels

Wei Cao[a,1], Xiao-Qiang Li[a,1], Xiang Wang[b,1], Tao Li[c], Xi Chen[a], Shui-Bing Liu[a], Qi-Bing Mei[a,*]

[a] *Department of Pharmacology, School of Pharmacy, The Fourth Military Medical University, 169 West Changle Road, Xi'an, Shaanxi 710032, China*
[b] *Department of Pathophysiology, School of Basic Medicine, Xi'an Medical University, Xi'an, Shaanxi 710021, China*
[c] *Forensic Department, School of Medicine, Xi'an Jiaotong University, Xi'an, Shaanxi 710061, China*

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ABSTRACT

In this study, three acidic polysaccharides (APS-3a, APS-3b and APS-3c) were obtained from *Angelica sinensis* (Oliv.) Diels. They displayed different structural features and anti-tumor activities. APS-3b and APS-3c significantly inhibited the growth of S180 tumors and increased the life spans of S180 tumor-bearing mice, whereas APS-3a had no significant effect. Further experiments showed that APS-3b and APS-3c could cause a concentration-dependent proliferation of the splenocytes, up-regulate IFN-γ, IL-2 and IL-6 mRNA expressions in splenocytes and stimulate the productions of NO and TNF-α in peritoneal macrophages. Taken together, the three acidic polysaccharides displayed different anti-tumor activities which were associated with their different structural characteristics.

1. Introduction

Cancer as one of the leading causes of death has threatened mankind for centuries. Although the efficacy of western medical treatments for cancer, including surgery, radiotherapy, chemotherapy for the majority of cancer types has been improved during the last three decades, producing severe adverse effects and increasing tolerance by cancers over time are still formidable problems in clinical medicine [1]. Therefore, some attentions have shifted to the discovery of new anti-cancer strategy, such as the use of oriental medicine. Since the discovery that Letinan, a polysaccharide from *Lentinus edodes* (Berk.) Sing inhibited mouse sarcoma 180 and displayed very low toxicity compared with chemical anti-tumor drugs [2,3], a number of polysaccharides with anti-tumor activity have been reported. Previous research showed that the structure of the polysaccharide is strongly related to its anti-tumor activity and suggested that the (1 → 3)-β-glucan with the (1 → 6)-β-glucan branches was important for the anti-tumor activity [4]. However, recently, some acidic polysaccharides displaying anti-tumor activity have been reported, such as the acidic polysaccharides isolated from *Phellinus linteus*, *Enteromorpha intestinalis* and Panax ginseng [5–7].

The root of *Angelica sinensis* (Oliv.) Diels (Chinese Danggui), a well-known herbal medicine, has been used historically in gynecology for thousands of years [8]. The polysaccharide as one of the main compounds in *A. sinensis* has also attracted much attention. In the last few years, the structures and anti-tumor effects of several neutral polysaccharides isolated from *A. sinensis* have been reported [9–14]. For example, we obtained a water-soluble polysaccharide from *A. sinensis* which had a repeating unit consisting of (1 → 4)-α-D-glucopyranosyl residue and (1 → 6)-α-D-glucopyranosyl residue in a molar ratio of 4:1 [9]. Moreover, Yamada et al. [14] reported a water-soluble polysaccharide isolated from *Angelica acutiloba* which was a (1 → 4)-α-D-glucan having side chains at O-6 of the glucosyl residues of the main chain. However, to our knowledge, there were few reports on the acidic *A. sinensis* polysaccharides and their anti-tumor activities.

So in the present study, we extracted the polysaccharides from *A. sinensis*, purified the acidic fractions by chromatography, obtained three acidic homogeneous polysaccharides designated as APS-3a, APS-3b and APS-3c, and further determined their chemical features. The *in vivo* therapeutic efficacies and *in vitro* cytotoxic activities of them on the S180 tumors were studied. Moreover, to further elucidate the underlying mechanisms of the anti-tumor activities, the effects of three acidic polysaccharides on splenocytes and

* Corresponding author. Tel.: +86 29 8477 4552; fax: +86 29 8477 4552.
E-mail address: qbmmei@gmail.com (Q.-B. Mei).
[1] These authors contributed equally to this paper.

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macrophages, two major populations of cells in the host defense system were also studied.

2. Materials and methods

2.1. Materials

The roots of *A. sinensis* were collected in Minxian County, Gansu Province, China. The coarse powder of the roots was air-dried in the shade and stored in a well-closed vessel for use.

T-series Dextran, DEAE-Sephadex A-25, Sephadex G-100, and Sephacryl S-400 were purchased from Amersham biosciences (Uppsala, Sweden). Trifluoroacetic acid (TFA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), concanavalin A (ConA), lipopolysaccharide (LPS, *Escherichia coli* serotype 026:B6) and dimethyl sulfoxide (DMSO) were purchased from Sigma (St. Louis, MO, USA). RPMI 1640, phosphate-buffered saline (PBS), fetal bovine serum and Trizol reagent were purchased from Gibco (Grand Island, NY, USA). Superscript III reverse transcriptase was purchased from Invitrogen (Gaithersburg, MD, USA). dNTP mixture, Tag DNA polymerase and RNAase inhibitor were obtained from Takara Bio Inc. (Otsu, Shiga, Japan). IFN-γ, IL-2, IL-4, IL-6, tumor necrosis factor-α (TNF-α) enzyme-linked immunosorbent assay (ELISA) kits were purchased from R&D Systems Inc. (Minneapolis, MN, USA). Cyclophosphamid (Cy) was purchased from Hengrui Medicament (Jiangsu, China). All the other chemical reagents were analytical reagent grade.

2.2. Extraction and fractionation of the polysaccharides

The isolation and purification protocols of crude polysaccharide were based on our previous published works [9,10]. Briefly, the powdered roots (2.0 kg) were extracted three times with ethanol at 80 °C for 3 h and the residue was decocted three times with water at 100 °C for 4 h. After centrifugation (3000 rpm, 20 min), the aqueous extract was concentrated at 60 °C in vacuum and treated with three volumes of ethanol for precipitation at 4 °C overnight. The gel-like precipitate was suspended in water and dialysed against distilled water (exclusion limit 3.5 kDa). The non-dialysable portion was frozen at −20 °C, then thawed and centrifuged again to remove insoluble materials. After the freeze–thaw process was repeated eight times, the supernatant was lyophilized and the crude polysaccharide fraction was obtained (APS, yield: 45.0 g).

APS (5%) was dissolved in distilled water and loaded onto a DEAE-Sephadex A-25 column (90 cm × 5 cm). The column was successively eluted with distilled water, 0.3 M and 0.5 M NaCl, each time until the disappearance in elute of positive reaction for carbohydrate by the phenol–sulfuric acid method [15]. The 0.5 M NaCl-eluted fraction was collected, dialysed, lyophilized and designated as APS-3.

APS-3 was further fractionated on a column (100 cm × 3.5 cm) of Sephacryl S-400, eluted with 0.1 M NaCl. After fractions of 10 ml were collected and monitored for the presence of carbohydrate using phenol–sulfuric acid assay, the eluted solution was separated into three sub-fractions (APS-3a, APS-3b and APS-3c).

The sub-fractions containing carbohydrate were pooled, dialysed and lyophilized, respectively. Each fraction (APS-3a, APS-3b and APS-3c) was further applied onto the column of Sephadex G-100 (100 cm × 3.5 cm), to get purified polysaccharides. The relevant fraction was concentrated, dialysed and lyophilized.

2.3. Chemical and structural analysis

2.3.1. Homogeneity and molecular weight

The homogeneity and molecular weights of the polysaccharides were evaluated and determined by high performance size exclusion chromatography (HPSEC) using a Waters Alliance 2690 instrument equipped with a tandem of a Shodex sb-803HQ (Showa Denkko, 8 mm × 30 cm) and a Biosep SEC-S3000 (Phenomenex, 7. 8 mm × 30 cm) column, eluted with 0.05 M Na_2SO_4 at a flow rate of 0.8 ml/min. The elution was monitored by Waters Alliance 2414 RI detector and the data were analyzed with Millennium 32 (Waters Alliance) software. The columns were calibrated with standard T-series Dextran T-130, T-80, T-40, T-20 and T-10.

2.3.2. Carbohydrate, uronic acid, protein content and optical rotation analysis

Neutral carbohydrate was quantified by the phenol–sulfuric acid method, using D-glucose as the standard [15]. Protein contents were measured by the Bradford [16] method using bovine serum albumin as standard. The uronic acid contents were determined by photometry with carbazole in the presence of sulfuric acid, using D-galacturonic acid as standard [17]. Optical rotations were measured using a Perkin-Elmer 343 polarimeter. Each sample was analyzed three times by the methods described above.

2.3.3. Monosaccharide analysis

Three polysaccharides were hydrolyzed with 2 M TFA and converted to their alditol acetates as previously described [18,19]. The resulting alditol acetates were analyzed by gas chromatography (GC) using an Agilent 6890N instrument fitted with FID and equipped with SE-54 column (30 m × 0.32 mm × 0.25 μm). The temperature of the column was kept at 160 °C for 10 min and then increased to 280 °C at the rate of 5 °C/min. The rate of N_2 carrier gas was 1.2 ml/min.

2.3.4. Determination of endotoxin contamination

LPS contamination was tested by Limulus amebocytes lysate (LAL) assay. In brief, 0.1 ml of samples (5 mg/ml), standards or endotoxin-free water was mixed with 0.1 ml of LAL for 1 h at 37 °C and observed for gelation.

2.4. Animals

Male ICR mice (weighed 19.0 ± 2.0 g) purchased from Shaanxi Academy of Traditional Chinese Medicine (SATCM) (Shaanxi, China) and BALB/c mice (5–7 weeks old), obtained from the Experimental Animal Center, the Fourth Military Medical University were kept in our animal facility for at least 1 week before use. All the animals were housed five per plastic cages with wood chip bedding in an animal room with 12/12 h light–dark cycle and allowed free access to standard mouse chow and water. The experimental procedures were in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals.

2.5. Cell lines

The ascites form of the sarcoma line S180 was acquired from SATCM. The cells were passaged through ICR mice before initiating an experiment. Seven days after the tumor cells injection, ascites cells from a single animal were harvested and used for the experiments *in vivo*.

2.6. In vivo anti-tumor test

Under sterile condition, S180 cells were washed three times with sterilized PBS and subcutaneously inoculated (0.2 ml, 2×10^6 cells/mouse) into the right armpits of ICR mice at day 0. The inoculated mice were divided into twelve groups (eight mice in each group), the normal group, S180-bearing group (model group), Cy group (positive control group) and nine polysaccharide-treated